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The Effect of X4 and R5 HIV-1 on C, C-C, and C-X-C Chemokines during the Early Stages of Infection in Human PBMCs

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To better define a mechanism underlying the increase in expression of certain proinflammatory chemokines during HIV-1 infection, we analyzed the effect of X4 HIV-1 infection on C, C-C, and C-X-C chemokine mRNA levels. We demonstrate that X4 HIV-1 infection augments the expression of RANTES, IP-10, MCP-1, and Ltn in peripheral blood mononuclear cells (PBMCs). R5 HIV-1 also induces an increase in both IP-10 and MCP-1 production. Binding of UV-inactivated HIV-1 elevates MCP-1, RANTES, MIP-1α, MIP-1β, and IL-8 expression, but fails to alter the production of IP-10, suggesting that the induction of IP-10 is dependent on downstream events following viral internalization. Indeed, recombinant gp120 alone was able to stimulate an eightfold increase in MCP-1 expression, but was unable to induce any detectable increase in IP-10 protein. HIV-induced modulation of chemokine expression suggests a mechanism by which HIV-infected monocytes and T cells might recruit target cells to sites of active viral replication, thus potentially aiding in the spread of the virus.

Key Words: HIV-1; chemokine; gp120.

INTRODUCTION

Cytokines play a pivotal role in the regulation and function of immune cells during viral infection (for review, see Poli *et al.,* 1995). Chronic immune activation during HIV infection, as well as HIV-specific enhancement of expression of certain cytokines, results in the dysregulation of the cytokine network (Poli *et al.,* 1995). For example, HIV infection is associated with an increase in the production of proinflammatory cytokines, especially during the later stages of disease (Poli *et al.,* 1995). High levels of TNF-α, IL-1β, and IFNγ are secreted by cultured peripheral blood mononuclear cells (PBMCs) (Hober *et al.,* 1989; Roux-Lombard *et al.,* 1989; Voth *et al.,* 1990; Vyakarnam *et al.,* 1991), while elevated levels of TNF-α are produced by macrophages (Antinori *et al.,* 1992) isolated from HIV-infected donors. Elevated levels of TNF-α, IL-6, and IL-1β have been detected in the serum (Arditi *et al.,* 1991; Breen *et al.,* 1990), IL-1β and IL-6 in the cerebrospinal fluid (Gallo *et al.,* 1989), TNF-α and IL-1β in the intestinal mucosa (Kotler *et al.,* 1993), and IFNγ in the lymph nodes (Boyle *et al.,* 1993; Emilie *et al.,* 1990) of HIV-infected individuals. The alteration of the cytokine network observed during HIV infection also includes the progressive loss of the ability to produce immunoregulatory cytokines, such as IL-2 and IL-12, which are critical for effective cell-mediated immune responses (Clerici *et al.,* 1993; Schulick *et al.,* 1993). Alterations in the balance of cytokine production may directly contribute to HIV pathogenesis by further stimulating HIV replication, by suppressing the ability of the immune system to mount a strong antiviral response, or by inducing cytokine-mediated cytopathic effects (Fauci, 1996).

While the aforementioned cytokines play a pivotal role in regulating the progression of HIV infection, the role of chemokines in the transmission and susceptibility to HIV infection is less well understood. Although many chemokines have been studied in the context of suppression of HIV replication, their individual contributions to the recruitment of leukocytes into the brain and peripheral immune organs during disease progression have not been fully defined. Monocytes have been proposed to play an important role in primary HIV infection, serving as an important target for R5 strains of HIV both during the initial stages of infection and during entry into the central nervous system (Koenig *et al.,* 1986; Roos *et al.,* 1992; Zhu *et al.,* 1993). The emergence of X4 HIV strains later in the course of the disease generally correlates with $CD4^+$ T cell decline and, finally, the development of AIDS (Cheng-Mayer *et al.,* 1991; Tersmette *et al.,* 1989). We wanted to determine whether X4 and R5 HIV-1 infection of PBMCs could result in altered production of chemokines, thus potentially selectively recruiting certain cellular targets during progression of HIV-1 disease. Our results reported here show that both R5 and X4 HIV-1-infected PBMCs exhibit increased production of C, C-C, and C-X-C chemokines, including monocyte chemotactic

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protein (MCP)-1, a potent activator of monocyte function and a major inducer of monocyte recruitment, and IFNγ-inducible protein (IP)-10, a major chemoattractant of activated T cells. Moreover, in activated PBMCs, X4 HIV-1 significantly enhances the expression of lymphotactin (Ltn). Interestingly, we show here that X4 HIV-1 infection exerts its effects on MCP-1 and IP-10 through two distinct and separate mechanisms. The induction of MCP-1 by X4 HIV-1 is mediated by events which occur as a part of virus binding to the cell surface, while events which are further "downstream" of virus infection result in an increase in IP-10 expression.

RESULTS

Infection with a X4 strain of HIV-1 results in a significant increase in the levels of RANTES, IP-10, and MCP-1 mRNA

To determine whether HIV-1 infection alters chemokine production in nonactivated PBMCs, cells from five donors were isolated and infected with the IIIB (X4) strain of HIV. We have previously shown (Wetzel *et al.,* 2000) that during 24–96 h in culture, chemokine transcripts can be detected at 24 h, and peak at 48 and 72 h in culture. Therefore, total RNA was isolated 48 h after infection, and the levels of chemokine transcripts were determined by RNAse protection analysis. Examination of chemokine levels from each of five donors revealed that infection of nonactivated PBMCs with an X4 strain of HIV-1 resulted in an elevation in RANTES, IP-10, and MCP-1 expression (Figs. 1 and 2), while levels of MIP-1α, MIP-1β, and IL-8 showed only a modest enhancement. Quantitation of the RNase protection analysis (Fig. 2) shows that X4 HIV-1 infection of non-PHA-activated PBMCs resulted in a modest 1.5-fold increase in RANTES mRNA levels and a threefold increase in MCP-1 mRNA levels (Fig. 1, lanes 1 and 2, and Figs. 2D and 2G, respectively). Interestingly, IP-10 expression was detected by non-PHA-activated PBMCs after infection with an X4 HIV-1 strain (Fig. 1, lanes 1 and 2; Fig. 2C). In contrast, a nonsignificant elevation of MIP-1α, MIP-1β, and IL-8 mRNA levels was observed after X4 HIV-IIIB infection of non-PHA-activated PBMCs (Fig. 1, lane 2, and Figs. 2F, 2E, and 2B, respectively). In addition, Ltn mRNA expression by nonactivated PBMCs was not detected in any of the five donors examined (Fig. 1, lanes 1 and 2; Fig. 2A), and X4 HIV-1 infection did not induce its expression (Fig. 1, lane 2; Fig. 2A).

These results show that nonactivated cells respond to HIV infection by selectively increasing the expression of certain chemokines. We next attempted to determine whether the nonactivated cells in these studies could be productively infected with X4 HIV-1. Nonactivated, HLA-DR-negative PBMCs were obtained by fluorescence-activated cell sorting and were subjected to infection with HIV-IIIB. We observed that nonactivated PBMCs produced modest, but detectable, HIV-1 p24 protein as early as 24 h postinfection (11 ng/ml at 24 h, 16.4 ng/ml at 48 h, and 21.3 ng/ml at 72 h postinfection). Moreover, nonactivated cells also clearly expressed the mature HIV-1 coreceptors. Specifically, 43% of the HLA-DR-/CD3$^+$ subpopulation expressed CXCR4, while 5% of the HLA-DR-/CD14$^+$ subpopulation expressed CXCR4. Thus, the elevated levels of MCP-1, RANTES, and IP-10 transcripts shown above (Figs. 1 and 2) appear to be associated with HIV-1 infection of the nonactivated PBMCs.

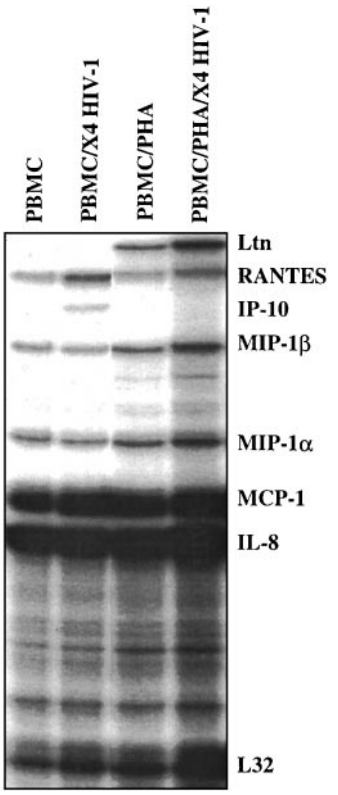

FIG. 1. The effect of X4 HIV-1 infection on chemokine mRNA levels in PBMCs. PBMCs were cultured with medium alone or activated with PHA (5 μg/ml), and 24 h later infected with X4 HIV-1 (strain IIIB). Forty-eight hours after infection, the level of chemokine mRNA was determined by RNAse protection assay. Results are presented as individual protected fragments for Ltn, RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, IL-8, and the housekeeping gene, L32. Autoradiogram shows the effect of X4 HIV-1 on chemokine production by nonactivated PBMCs and shows a representative of the result from one of five different donors for nonactivated PBMCs or seven different donors for PHA-activated PBMCs.

Activation state of the target cells may also play a role in the outcome of chemokine production, since various cell types and activation states produce distinct chemokine profiles. To test this hypothesis, experiments were carried out to determine the effect of X4 HIV-1 infection of activated PBMCs on chemokine expression. Cells were activated with PHA for 24 h, followed by X4 HIV-1 infection. Forty-eight hours after infection, chemokine transcript levels were measured by RNAse protection analysis (Fig. 1, lanes 3 and 4; Fig. 2). In each of seven donors

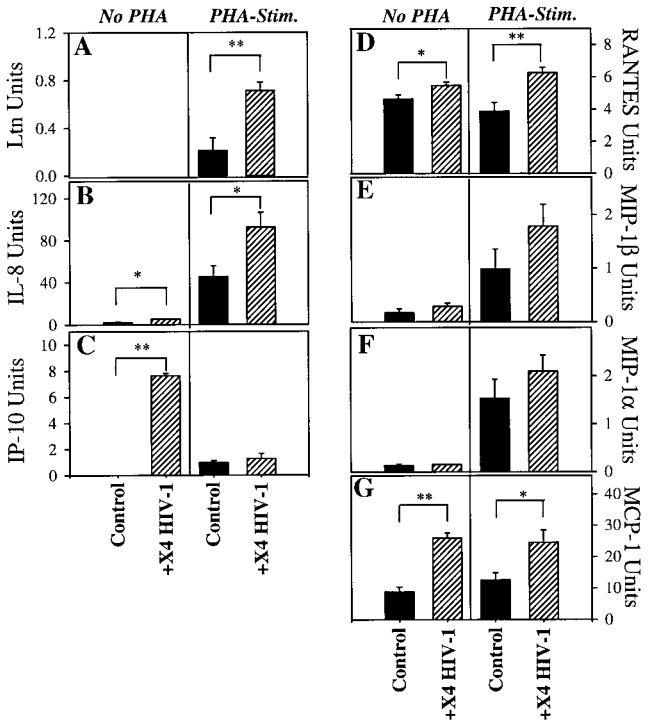

FIG. 2. The effect of X4 HIV-1 infection on chemokine mRNA levels in PBMCs. Cells were cultured with medium alone or activated with PHA (5 μg/ml), and 24 h later infected with X4 HIV-1 (strain IIIB). Forty-eight hours after infection, the level of chemokine mRNA was determined by RNAse protection assay. Quantitation of chemokines is presented as the mean (±SD) of triplicate mRNA samples normalized from a single donor to the housekeeping gene, L32. Values are representative of the results from seven different donors. Solid bars represent uninfected cells, and hatch bars represent X4 HIV-1-infected cells. $^{*}P < 0.05$; $^{**}P < 0.005$.

examined, PHA activation alone significantly elevated Ltn, IL-8, MIP-1α, and MIP-1β chemokine mRNA levels, while RANTES and MCP-1 mRNA expression was not altered after activation (Fig. 1, lane 3; Fig. 2). In four of seven donors analyzed, IP-10 transcripts were not detected in PBMCs 24 h after PHA activation (Fig. 1, lane 3). However, in those donors expressing detectable constitutive IP-10, the X4 HIV-1 infection did not alter the expression of this chemokine (Fig. 2C). Interestingly, Ltn mRNA expression was detected after activation in each of seven donors examined, and X4 HIV infection increased Ltn levels 3.5-fold (Fig. 1, lanes 3 and 4; Fig. 2A). Moreover, both RANTES and MCP-1 mRNA production was augmented after X4 HIV-1 infection of activated cells by twofold (Fig. 1, lane 4; Figs. 2D and 2G). X4 HIV-1-infection of activated PBMCs did not result in a significant change in MIP-1α or MIP-1β (Fig. 1, lanes 3 and 4; Figs. 2E and 2F), a result which is consistent with the data from infection of nonactivated cells. In contrast to the nonactivated PBMCs, X4 HIV-1-infected activated cells produced a twofold greater level of IL-8 than uninfected cells (Fig. 1, lanes 3 and 4; Fig. 2B). These data suggest that X4 HIV-1 infection of activated PBMCs results in a significant increase in the levels of Ltn, IL-8, RANTES, and MCP-1.

Infection with X4 HIV-1 results in an increase in RANTES, IP-10, and MCP-1 protein levels in nonactivated PBMCs

X4 infection of PBMCs resulted in significant changes in IP-10, RANTES, and MCP-1 mRNA expression in all donors. Therefore, we examined the effect of X4 HIV-1 infection on the expression of MCP-1, RANTES, and IP-10 protein levels. Non-PHA-activated and PHA-activated PBMCs were infected with X4 HIV-1, and chemokine protein levels were determined by ELISA. In non-PHA-activated cells, HIV infection significantly increased the expression of MCP-1, RANTES, and IP-10 protein levels 48 h after infection (Fig. 3), consistent with results obtained at the mRNA level. In contrast, our results show that X4 HIV infection had no effect on MCP-1 protein expression by activated cells (Fig. 3A). The basal level of

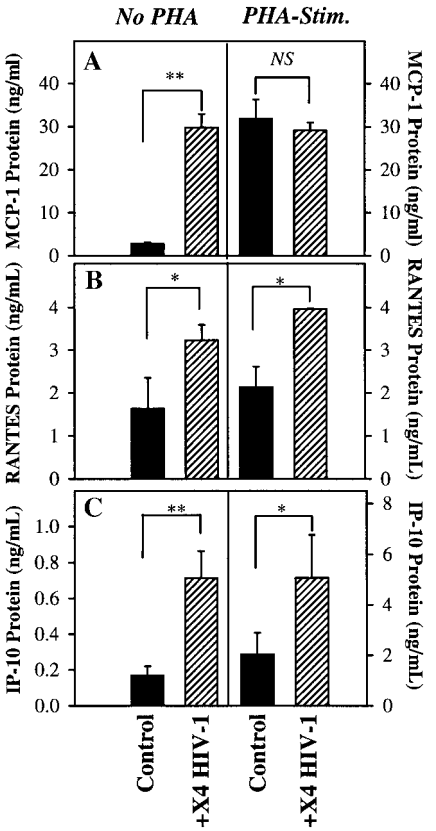

FIG. 3. Effect of X4 HIV-1 on RANTES, IP-10, and MCP-1 protein levels expressed by non-PHA-activated and PHA-activated PBMCs. Cells were cultured with medium alone or activated with PHA (5 μg/ml), and 24 h later, designated cultures were infected with X4 HIV-1 (strain IIIB). Forty-eight hours after infection, supernatants were removed, and the concentrations of MCP-1 (A), RANTES (B), and IP-10 (C) protein were determined for noninfected (solid bars) and infected (hatched bars) groups. Values represent the mean (±SD) of the triplicate cultures and are representative of results from seven different donors. $^{*}P < 0.05$; $^{**}P < 0.005$. NS, not significant.

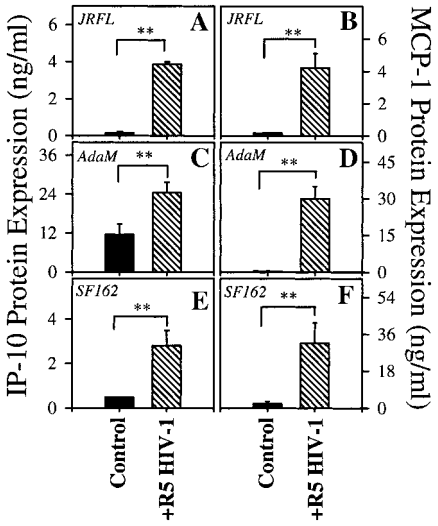

FIG. 4. Effect of R5 HIV-1 infection on the production of IP-10 and MCP-1 protein levels expressed by non-PHA-activated PBMCs. Cells were cultured for 24 h followed by HIV infection with either R5 HIV-1 strain JRFL, AdaM, or SF162. Forty-eight hours after infection, the concentrations of IP-10 (A, C, and E) and MCP-1 (B, D, and F) protein were determined for both noninfected (solid bars) and infected (hatched bars) cultures. Values represent the mean (±SD) of triplicate cultures and are representative of results from seven different donors. $^{**}P < 0.005$.

MCP-1 protein is increased following activation alone, so that any further induction by X4 infection may be masked by the activation process. On the other hand, our results showed that RANTES and IP-10 protein levels are significantly elevated by infection in activated cells (Figs. 3B and 3C). Interestingly, only three of seven donors expressed detectable levels of IP-10 mRNA after activation of PBMCs (Fig. 1, lane 3; Fig. 2C), while at the protein level, all donors expressed IP-10 after PHA stimulation (Fig. 3C).

IP-10 and MCP-1, but not RANTES, protein levels are selectively increased following infection with R5 HIV-1

It has been proposed that the cellular tropism of HIV-1 is largely responsible for establishing a primary infection (Roos *et al.,* 1992; Zhu *et al.,* 1993). Therefore, we also examined the effect of infection with R5 strains on the production of chemokines. Cells were infected with the R5 HIV-1 strains JRFL, AdaM, or SF162, and levels of RANTES, IP-10, and MCP-1 were determined. Our results (Fig. 4) show that both IP-10 and MCP-1 expression was significantly elevated following infection with each of the three R5 strains. In contrast, accumulated results from several experiments showed that there were no significant changes in RANTES expression after R5 HIV infection (data not shown). Interestingly, we found that R5 HIV-1 infection of nonactivated PBMCs resulted in a significant increase in IP-10 and MCP-1 expression. Therefore, we examined the cell surface expression of the R5 HIV-1 coreceptor, CCR5, on freshly isolated PBMCs from three different donors and found that 3–12% of the total population express CCR5 (data not shown). Moreover, elevated IP-10 and MCP-1 protein levels were observed in nonactivated PBMCs 48 h after infection, at a time when 2–20% of the cells are positive for CCR5 (based on results from five different donors).

UV irradiation of X4 HIV-1 inhibits the induction of IP-10 mRNA and protein levels, whereas MCP-1 mRNA and protein expression remains unchanged

Based on our earlier observation (Figs. 1 and 2) that X4 HIV-1 increases IP-10 and MCP-1 production, we carried out experiments to determine whether viral binding was sufficient to induce MCP-1 and/or IP-10 expression. UV-irradiated (nonreplicating) or nonirradiated (replicative competent) X4 HIV-1 was added to PBMCs, and chemokine expression was assessed. Our results show that treatment with UV-irradiated virus resulted in a 21-fold increase in MCP-1 protein expression (Fig. 5A). In contrast, the inactivated X4 HIV failed to induce a significant increase in IP-10 expression (Fig. 5B). Our results also show that MCP-1 mRNA levels were increased after treatment with irradiated HIV-1; however, the irradiated virus failed to induce an increase in IP-10 mRNA (data not shown). Interestingly, RANTES, MIP-1α, MIP-1β, and IL-8 mRNA levels expressed by nonactivated PBMCs were also elevated after treatment with irradiated virus (data not shown). These results suggest a mechanism by

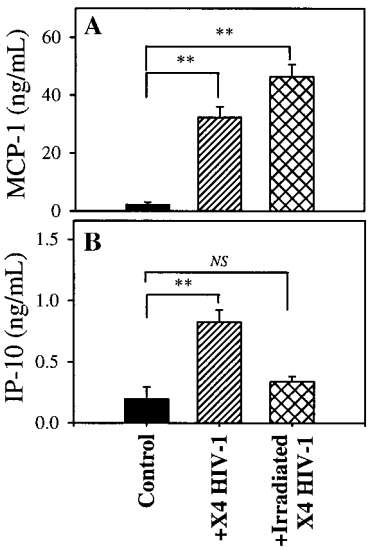

FIG. 5. UV-irradiated X4 HIV-1 (nonreplicating) enhances MCP-1 protein; however, it has no effect on IP-10 expression. PBMCs were cultured for 24 h and then infected with X4 HIV-1 (strain IIIB) (hatched bars) or with UV-irradiated virus (crosshatched bars). Forty-eight hours after infection, MCP-1 (A) and IP-10 (B) protein levels were determined by ELISA. Values represent the mean (±SD) of the triplicate cultures and are representative of results from three different donors. $^{**}P < 0.005$. NS, not significant.

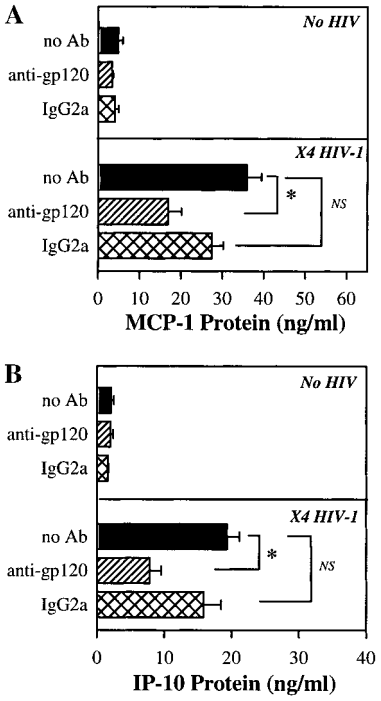

FIG. 6. Treatment with monoclonal anti-gp120 antibody inhibits the X4 HIV-1 induction of MCP-1 and IP-10 expression by non-PHA-activated PBMCs. Prior to infection, X4 HIV-1 (strain IIIB) was treated with either anti-gp120 (hatched bars) or the IGg2A isotype negative control (crosshatched bars) for 1 h at 37°C. The virus/antibody mixture was then added to the non-PHA-activated PBMCs for 2 h. The cells were washed, and fresh medium was replaced. Seventy-two hours after treatment, supernatants were collected, and MCP-1 (A) and IP-10 (B) protein expression was determined. Values represent the mean (±SD) of the triplicate cultures and are representative of results from three different donors. $*P < 0.05$. NS, not significant.

which X4 HIV-1 infection modulates chemokine production by PBMCs. Our data show that productive viral infection is required for the induction of IP-10 expression, while early events associated with viral binding appear to be sufficient for the induction of MCP-1, RANTES, MIP-1α, MIP-1β, and IL-8.

Neutralization of HIV-1 with anti-gp120 monoclonal antibody inhibited the virus-induced production of IP-10 and MCP-1 protein levels

To determine whether the X4 HIV-induction of MCP-1 is mediated through binding of HIV-gp120 to target cells, X4 HIV-1 virions were incubated with anti-gp120 and then added to PBMCs. The results show that treatment with anti-gp120 monoclonal antibody significantly reduced the ability of X4 HIV-1 to enhance either MCP-1 or IP-10 expression (Fig. 6). Treatment with isotype-matched control immunoglobulin consistently had no significant effect on the ability of HIV to induce the production of these chemokines. We interpret these results to indicate that anti-gp120 monoclonal antibody blocked MCP-1 expression by inhibiting viral binding (Fig. 6A). In addition, treatment with anti-gp120 monoclonal antibody inhibited IP-10 expression by blocking binding, thus inhibiting downstream replicative events (Fig. 6B). These data provide evidence that gp120-mediated viral binding is required for the induction of either MCP-1 or IP-10.

Recombinant HIVgp120 increases the production of MCP-1, while IP-10 levels remain unchanged

Based on the ability of UV-inactivated HIV to induce MCP-1 expression, we postulated that X4 HIV-1 binding alone to the cell surface is sufficient to induce MCP-1 expression. To further test this hypothesis, PBMCs were treated with recombinant gp120, and IP-10 and MCP-1 levels were determined. Our results (Fig. 7) show that treatment with gp120, at concentrations as low as 1 ng/ml, induced a significant increase in MCP-1 expression (Fig. 7A). As expected, gp120 administration at concentrations as high as 100 ng/ml failed to induce a detectable increase in IP-10 expression (Fig. 7B). It is apparent that X4 HIV-1-mediated stimulation of IP-10 expression requires viral internalization and other downstream events.

DISCUSSION

In this report, we present evidence that shows that during the first 48 h after infection with both X4 and R5 strains of HIV-1, there is a significant selective increase

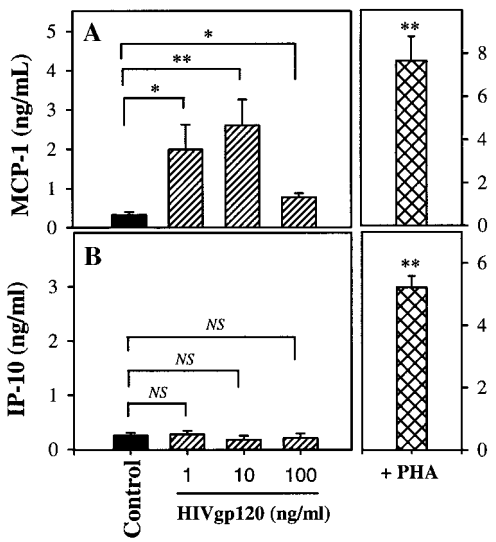

FIG. 7. Recombinant gp120 stimulates MCP-1 protein expression, but is unable to induce any detectable increase in IP-10 expression. After an initial 24 h in culture, non-PHA-activated PBMCs were treated with designated concentrations of HIV-gp120, and after 48 h, MCP-1 (A) and IP-10 (B) protein expression was determined. Chemokine expression by PHA-activated PBMCs is included as a positive control (crosshatched bars). Values represent the mean (±SD) of the triplicate cultures and are representative of results from three different donors. $*P < 0.05$; $**P < 0.005$. NS, not significant.

in the levels of the proinflammatory chemokines IP-10 and MCP-1, while RANTES expression is significantly augmented only by X4 strains. Moreover, our results (Fig. 1) show that X4 HIV-1 infection of nonactivated PBMCs induces a modest increase in MIP-1α, MIP-1β, and IL-8 mRNA transcripts. The present results are important in view of earlier studies showing that HIV-1 infection alters production of several cytokines, resulting in stimulation of viral replication, suppression of antiviral responses, and induction of cytokine-mediated cytopathic effects (Poli *et al.,* 1995). However, the clinical relevance of elevated levels of chemokines during HIV transmission and pathogenesis remains to be defined. In this regard, certain chemokines compete with HIV for shared coreceptors, but they also potentially play a crucial role in attracting noninfected target cells to the site of active viral infection. A recent report by Weiss *et al.* (1999) demonstrated an important role for astrocyte-derived MCP-1 in mediating migration of monocytes and activated lymphocytes across an *in vitro* model of the blood brain barrier and may support evidence that monocytic infiltration of the central nervous system is an important feature of AIDS-related neuropathology and a significant correlate of dementia (Glass *et al.,* 1995; Price *et al.,* 1988). In contrast with the aforementioned studies, our results suggest a mechanism by which HIV-1 infection modulates chemokine expression in the periphery, where monocytes and T cells are major cellular targets for HIV-1.

Interestingly, while Ltn expression was undetectable in nonactivated cells, X4 HIV-1 infection of activated cells resulted in an average threefold increase in the mRNA level. Our results are consistent with previous reports that Ltn mRNA expression in resting peripheral blood leukocytes is dependent on activation (Kennedy *et al.,* 1995; Hedrick and Zlotnik, 1998). In addition, recent studies suggest that some leukocytes, which play a role in the initial phases of the inflammatory response (mast cells, intraepithelial $\gamma\delta$ T cells, and NK cells), are capable of producing Ltn, and second, that the kinetics of Ltn expression upon activation are extremely rapid (Hedrick and Zlotnik, 1998). Finally, the types of cells that respond to Ltn are primarily NK cells, and both $CD4^+$ and $CD8^+$ T cells (Hedrick and Zlotnik, 1998; Giancarlo *et al.,* 1996). It can be postulated that Ltn may play a major role in the antiviral inflammatory response to HIV-1 infection.

A recent report by Greco *et al.* (1999) demonstrated that infection of $CD4^+$ T cells with R5 HIV-1 resulted in elevated levels of MIP-1α and MIP-1β following a 2-week culture period; however, these investigators found that infection with X4 HIV-1 had either no effect or resulted in a decrease in the production of either MIP-1α or MIP-1β. Interestingly, infection with either of the viral phenotypes led to an increase in RANTES production. Similarly, Schmidtmayerova *et al.* (1996) observed the induction of MIP-1α and MIP-1β expression in human monocytes following infection with R5 virus. Based on the reported correlation between HIV-1 replication and MCP-1 levels in the cerebrospinal fluids of AIDS patients with HIV encephalitis, Mengozzi *et al.* (1999) found that MCP-1 secretion and expression were consistently upregulated over constitutive levels in monocyte-derived macrophages infected with R5 HIV-1. These data, taken together with our findings, support the notion that HIV-1 directly regulates the expression of selected chemokines. The data presented in this article suggest a mechanism by which HIV-1-infected cells might recruit uninfected potential target cells to the site of active viral replication, thus aiding in the spread of the virus.

The increase in chemokine expression has the potential to significantly alter the immune response to the virus. Several recent reports have shown that certain chemokines can enhance HIV infection (Kelly *et al.,* 1998; Kinter *et al.,* 1998; Morinchi *et al.,* 1998). Experiments carried out with RANTES, MIP-1α, MIP-1β, and MCP-1 show enhanced X4 HIV infection when added 48 h prior to infection, but lose this activity when added at the time of infection (Dolei *et al.,* 1998). Several explanations for these results have been suggested. For example, some of these effects were associated with increased levels of CXCR4 transcripts and with increased T cell proliferation (Dolei *et al.,* 1998). More recently, results show that pretreatment of monocytes and monocyte-derived macrophages with MCP-2 substantially increases R5 HIV replication (Kelly *et al.,* 1998). On the other hand, a proposed mechanism for RANTES-induced HIV-1 replication has emerged from recent studies (Trkola *et al.,* 1999), which have shown that RANTES oligomers bind to glycosaminoglycans on both the virion and the cell membranes, activating a tyrosine-kinase-dependent signal that enhances viral infectivity. In addition, it is known that certain chemokines can activate T cells and induce IL-2 production (Price *et al.,* 1988), along with other cell-activating cytokines such as IL-1, IL-2, and TNF-α, which elevate HIV replication (Poli *et al.,* 1994).

Results reported here show that infection with X4 HIV-1 results in an increase in RANTES, MIP-1α, MIP-1β, IL-8, and most profoundly, MCP-1 and IP-10. We examined the mechanism by which HIV infection mediates induction of MCP-1 and IP-10 expression and compared the ability of irradiated and nonirradiated X4 HIV-1 to induce chemokine expression. The UV irradiation does not interfere with the process of target cell binding and fusion, but renders the virus unable to replicate (Henderson *et al.,* 1992). Our results show that irradiated X4 virus retains the ability to induce MCP-1 expression, but was unable to induce a detectable increase in the expression of IP-10. These results suggest that productive viral infection is required for the induction of IP-10 expression, while early events associated with viral binding are most likely responsible for the induction of MCP-1. A slight increase in the ability of UV-irradiated HIV-1 to induce

MCP-1 may be associated with inhibition of cytopathic effects. In addition, we also show that the HIV-1 gp120 induces a significant increase in MCP-1 but not IP-10 expression. These results provide further evidence that viral gp120 binding to cell surface receptors plays a major role in mediating the elevation in the level of MCP-1. Recent studies suggest that the viral envelope first interacts with the cell surface through the binding of the gp120 subunit with CD4, followed by a conformational change in the envelope, and then an additional binding interaction with the coreceptor (Loetscher *et al.,* 2000). The formation of this trimeric complex may expose a region of the envelope protein, which promotes the fusion of the viral envelope, to the cell membrane. These events may be sufficient to induce signal transduction and transcriptional activation without viral replication. Indeed, recent studies have shown that IFNγ, IL-10, and IL-12 cytokines are elevated following HIV-1 infection or treatment with HIV-1 gp120 in PBMCs and macrophages (Gessani *et al.,* 1997). Finally, we show here that neutralizing the virus with anti-gp120 blocks viral binding (inhibiting the induction of MCP-1), and further downstream, viral replicative events (inhibiting the induction of IP-10). The role of viral transcriptional transactivating regulators, such as tat, nef, vpr, and rev, that might be involved in the induction of IP-10, is currently under investigation. Moreover, studies to address the mechanism by which R5 HIV-1 modulates IP-10 and MCP-1 are ongoing in our laboratories.

In summary, our data show that both R5 and X4 HIV-1 strains induced the production of mRNA transcripts for, and protein expression of, MCP-1 and IP-10 during the early stages of infection. The physiological consequence of enhanced levels of MCP-1, a potent activator of monocyte function and a major inducer of monocyte recruitment, could potentially attract noninfected target cells to the site of active viral replication. Interestingly, the role of IP-10 during HIV-infection and pathogenesis remains undefined; however, elevated levels of IP-10, a major chemoattractant for activated T cells, may result in the recruitment of a larger number of potential $CD4^+$ T cells as HIV-1 targets. The influx of $CD4^+$ T cells would aid in the switch from the early, asymptomatic R5 phase to later X4 stages of the disease, correlating with a $CD4^+$ T cell decline and progression to AIDS. The capacity of HIV infection to selectively enhance proinflammatory chemokine expression during the initial stages of infection suggests a mechanism by which HIV-1 may control host cellular immune factors, potentially aiding in the progression of disease. Importantly, our data provide additional evidence for two distinct mechanisms by which HIV-1 exerts its effects on MCP-1 and IP-10 expression in PBMCs during the initial stages of infection.

MATERIALS AND METHODS

Isolation of PBMCs and culture

PBMCs were obtained from the whole blood of normal donors and isolated by Ficoll–Paque Plus (Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation. Isolated PBMCs were plated at a cell density of 2×10^6 cells/ml in 24-well tissue culture plates. Cell cultures were maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated, low-endotoxin fetal calf serum (Hyclone, Logan, Utah), 10 μg/ml gentamicin reagent solution (Life Technologies), and 1 mM L-glutamine (Life Technologies). Designated cultures of PBMCs were treated with HIV-1 gp120 from the HIV-1 MN X4 tropic strain (NCI, Frederick, MD), using the culture conditions described above. PBMCs were cultured in the presence or absence of T cell mitogen, phytohemagglutinin (PHA), at 5 μg/ml for 18 h. Supernatants were removed 48 h after HIV-1 gp120 administration, and protein ELISA was performed to determine protein levels of MCP-1 and IP-10.

Viruses

The X4 IIIB and R5 JRFL, AdaM, SF162 strains of HIV were obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program (Rockville, MD). The IIIB strain of HIV-1 was propagated in the human T cell line, Molt4-IIIB. The multiplicity of infection (m.o.i.) of the IIIB strain was determined by quantitation of syncytium formed by HIV-infected lymphocytes when cocultured with exponentially growing CD4-bearing SupT1 cells. All R5 strains of HIV were propagated in cultures of PBMCs from adult donors. Virus was concentrated from culture supernatants and purified by pelleting at 110,000*g* for 90 min. The pellets were gently washed and resuspended in medium. This procedure produces stock virus of between 10^6 and 10^7 syncytia-forming units per 0.1 ml. The 50% tissue culture infectious dose ($TCID_{50}$) for the R5 virus was determined using PBMCs. Briefly, graded doses of virus were inoculated onto PBMCs, and the extent of replication was measured every 3 days in the culture fluid by a p24 antigen ELISA. The final titer was calculated at the peak time of virus production.

HIV-1 infection of PBMCs

Designated cultures of PBMCs were treated with PHA (5 μg/ml) at a density of 2×10^6 cells/ml. After 24 h in culture, titered viral strains were resuspended in serum-free, low-endotoxin RPMI medium and then added to the PBMCs at an m.o.i. of 0.1. PHA-stimulated and nonstimulated PBMCs cultures were infected for 2 h at 37°C and washed, and 48 h after infection, cells and supernatant was harvested to determine chemokine mRNA or protein levels, respectively.

HIV-1 p24 antigen capture assay

Determination of HIV-1 p24 levels was carried out using an antigen capture assay. A kit for this assay was obtained from the AIDS Vaccine Program, Biological Products Laboratory at the Frederick Cancer Research and Development Center in Frederick, MD and is composed of 96-well plates coated with monoclonal anti-HIV-1 p24, a primary antibody (rabbit anti-HIV-1 p24 serum), and a secondary antibody (goat anti-rabbit IgG) (H + L) peroxidase-labeled antibody. Dilutions of the standards and samples were added to the plates and incubated at 37°C for 2 h. The plates were washed, and the primary antibody was added and incubated at 37°C for 1 h. The plates were washed, and the secondary antibody was added and incubated at 37°C for 1 h. The plates were washed, peroxidase substrate was added, and the reaction was stopped by adding $4NH_2SO_4$. The level of colored product was measured spectrophotometrically at a wavelength of 450 nm.

Cell sorting and FACS analysis of PBMCs

Freshly isolated PBMCs were washed using a buffer composed of Hanks' balanced salt solution supplemented with 2% endotoxin-free fetal bovine serum (HF buffer) and suspended at a density of 3.5×10^7 cells/ml. Cells were incubated with 2% goat serum at 4°C for 30 min. The cells were washed and suspended in 50 μl HF containing Cy-chrome-conjugated mouse anti-human HLA-DR (G46-6) (BD Pharmingen, San Diego, CA) for 1 h at 4°C. The HLA-DR-negative and -positive cells were separated by FACS, and the DR-negative population was either set aside for analysis of HIV-1 infectivity or the cells were subjected to further staining using either fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3 (UCHT1) or CD14 (M5E2) together with R-phycoerythrin (R-PE)-conjugated mouse anti-human CCR5 (2D7/CCR5) or CSCR4 (12G5). All flow cytometry was carried out using a Coulter EPICS XL flow cytometric analyzer (Coulter Corp., Hialeah, FL).

Measurement of chemokine mRNA by RNase protection analysis

The expression of chemokines was measured by RNase protection analysis using the RiboQuant Multi-Probe RNase Protection Assay System (Pharmingen). Briefly, 10 μg RNA from each sample, isolated using the RNAzol method (Tel-Test, Friendswood, TX), was allowed to hybridize in solution with the radiolabeled antisense RNA probe. The probe is generated to include an RNA probe set for the human chemokines Ltn, RANTES, MIP-1α, MIP-1β, IFNγ, IP-10, MCP-1, IL-8, and I-309 according to the manufacturer's instructions. The hybridized ^{32}P-labeled probe-transcript duplex was subjected to digestion with RNase, and the protected probes were purified and resolved on 5% denaturing polyacrylamide. The gels were then dried and exposed to a phosphorimaging screen, and protected fragments were visualized and quantitated using a Model GS-525 phosphorimager (Bio-Rad, Hercules, CA). In some cases, both short and long exposure times are analyzed to obtain accurate data from relatively abundant and nonabundant transcripts, respectively. Results are expressed as relative units, which are calculated after normalizing based on optical density of the L32 band for each lane.

Measurement of chemokine protein levels by ELISA

The concentration of chemokines present in culture supernatants was determined by ELISA, using matched mouse monoclonal or polyclonal antibody capture and detection antibodies in a sandwich ELISA. Anti-chemokine "capture" antibodies (Pharmingen) used in these experiments were rabbit polyclonal anti-human RANTES, monoclonal anti-human MCP-1 (clone 10F7), and monoclonal anti-human IP-10 (clone 4D5). The capture antibodies were coated onto plastic microwell plates (Nunc Maxisorb) and blocked with 1% BSA-containing PBS, and graded dilutions of culture supernatant or recombinant standard (Peprotech, Rock Hill, NJ) were added. After washing, the captured chemokine proteins were detected using biotin-conjugated anti-chemokine "detection" antibodies (Pharmingen), polyclonal anti-human RANTES, monoclonal anti-human MCP-1 (clone 5D3-F7), and monoclonal anti-human IP-10 (clone 6D4), followed by HRP-linked streptavidin. Following the addition of ABTS 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid) in buffer, the level of colored product was measured spectrophotometrically (405 nm).

UV irradiation of HIV strains

For the UV source, total emission from a 15-W GE15T8 low-pressure mercury germicidal lamp controlled by a rheostat was used. Incident (254 nm) UV dose rate was 5 J/m^2 for viral inactivation as determined by Latarjet dosimeter. The kinetics of UV inactivation of HIV-1 infectivity has been previously described (Henderson, 1992). One-milliliter aliquots of virus RPMI medium was placed in 60-mm petri dishes and exposed with gentle agitation to ensure uniform irradiation. Virus samples were kept on ice before and after irradiation. Virus inactivation was confirmed by syncytium assays. We observed no syncytium formation of irradiated virus.

Anti-gp120 assays and neutralization with anti-gp120 monoclonal antibody

Monoclonal anti-HIV gp120 MN was a generous gift of Dr. Oleg Chertov (NCI, Frederick, MD). The nonspecific antibody control was of the isotype IgG2a (Organon Teknika Corp., Durham, NC). Final antibody concentrations used in the experiments were 5 μg/ml, a 1:200

dilution of the stocks. In the HIV-induced chemokine blocking assays, both the anti-gp120 and the IGg2A isotype negative control were incubated with HIV-IIIB (X4) for 1 h at 37°C. The virus/antibody mixture was then added to PBMCs for 2 h. The cells were washed, and fresh media was replaced. Seventy-two hours after infection, supernatants were removed and MCP-1 and IP-10 protein ELISA was performed. The ability of HIV anti-gp120 MN to neutralize HIV-IIIB was confirmed by syncytium count as well as HIV P24 ELISA.

Statistics

The data are presented as the mean ± SD. Where appropriate, the statistical difference between experimental groups was assessed using the Student's *t* test.

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